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(54) Title: BLOOD-COAGULATION FACTOR XIIa β MONOCLONAL ANTIBODY AND IMMUNOASSAY

(57) Abstract

A monoclonal antibody which binds to Factor βXII and which shows substantially no binding to Factor XII. This antibody may be used in immunoassays to measure Factor βXIIa levels in blood, for example for studies of blood coagulation systems and of thrombotic disorders.

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BLOOD-COAGULATION FACTOR XIIa β MONOCLONAL ANTIBODY AND IMMUNOASSAY.

The present invention relates to an immunoassay and to reagents for such an assay. It relates in particular to blood coagulation Factor XII and activated forms thereof, Factor XIIa.

Factor XII is an inactive zymogen present in normal blood. Factor XII is readily converted, in the presence of kallikrein, high molecular weight kininogen and a negatively charged surface into a two chain form that is enzymatically active. This 80-Kd serine proteinase, often called Factor αXIIa, has a 52-Kd heavy chain linked by a disulphide bond to a 28-Kd light chain. Proteolysis of this factor releases a 40-Kd peptide from the heavy chain, and results in a product, Factor βXIIa, that retains serine protease activity, but in which the 28-Kd chain of Factor αXIIa is disulphide-linked to a small peptide fragment derived from the former 52-Kd heavy chain. In many cases the small peptide fragment has a molecular weight of 2000-d, but fragments of different size have been observed, for example, 800-d and 3000-d fragments.

The term "Factor XIIa", abbreviated to "XIIa" is used herein to denote any form of activated Factor XII, that is to say, any derivative of Factor XII having serine proteinase activity. It includes any form of XIIa

produced from Factor XII in vitro and any form occurring in vivo and obtainable from natural sources. It further includes any analogue of a naturally-occurring protein that has a modified amino acid sequence, for example, one or more so-called "conservative" changes to the amino acid sequence, that is to say, changes (additions, removals or substitutions) that do not affect the properties of the molecule, in particular the immunogenic and enzymatic properties. The term includes any synthetic copy and any synthetic analogue of a naturally occurring Factor XIIa, whether produced by chemical synthesis or by recombinant DNA technology. It includes any form of αXIIa and any form of βXIIa. The terms "βXIIa" and "Factor βXIIa", and "αXIIa" and "Factor αXIIa" are used analogously herein to denote any form of such a molecule.

The amino acid sequence of a form of Factor βXIIa having a 2000-d peptide fragment has been described (K. Fujikawa & B.A. McMullen, J. Biol. Chem. 258, 10924-10933, 1983), and speculations have been made as to its tertiary structure (D.E. Cool et al, J. Biol. Chem. 260, 13666-13676, 1985). The cleavage sites of this form of βXIIa have been defined further by Cool et al (J. Biol. Chem. 262, 13662-13672, 1987): αXIIa is stated to be produced from XII by cleavage between Arg^{353}-Val^{354}, and βXIIa is produced from αXIIa by cleavage between Arg^{334}-Asn^{335}, Arg^{343}-Leu^{344} and Arg^{353}-Val^{354}, resulting in two

polypeptide chains of 9 and 243 residues respectively.

Although the present invention is particularly concerned with human Factor XIIa it is not limited to the human protein or to a XIIa protein having any specific amino acid sequence (see Fujikawa & McMullen above).

It is known that elevated blood levels of cholesterol, Low Density Lipoprotein (LDL) and Apolipoprotein B are positively correlated with the long term mortality risk from Ischaemic Heart Disease (IHD) and Acute Myocardial Infarction (AMI). Similar positive correlations are found with lowered blood levels of High Density Lipoprotein (HDL) and Apolipoprotein A1.

None of these parameters, however, is usefully predictive of the risk of AMI in individuals. Meade et al from Northwick Park Hospital (Meade et al Lancet (i) 1050-4; Meade. Haemostasis 1983, 13, 178-85; Meade et al Lancet 1986, (ii) 533-7) and Kannel, Wolf, Castelli & Agostino of the Framingham Study (Kannel et al J.A.M.A. 1987, 258(9), 1183-6) have shown that, under carefully controlled conditions, measurements of fibrinogen and Factor VII coagulant activity can predict the likelihood of AMI better than can various lipid measurements. Factor VII itself is a single chain protein having low activity but it can be converted to a two chain form, Factor VIIa, which more active. Substances that can activate Factor VII to VIIa include Factor Xa, Factor IXa, Factor XIIa, thrombin.

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As indicated above, Factor VII activity has been shown under carefully controlled conditions to be useful in predicting the likelihood of AMI in individuals. However, currently available methods of measuring Factor VII activity are subject to large variations. In particular venepuncture, the method generally used to obtain blood samples, releases variable amounts of tissue factor which, as mentioned above, is involved in one method of activation of Factor VII. Accordingly the use of factor VII activity in predicting AMI is not sufficiently reliable for general use.

The present invention is based on the observation that an increased level of lipids in the diet increases Factor VII activity by increasing the steady state concentration of Factor XIIa, for example βXIIa. That is to say, Factor XIIa, for example βXIIa, is a link between hyperlipidaemia and Factor VII.

Accordingly, it is proposed to use measurements of levels of Factor XIIa, for example βXIIa, in plasma to predict the likelihood of heart disease, in particular, ischaemic heart diaease and acute myocardial infarction (AMI) in individuals, in analogy to the use of Factor VII activity, but avoiding the disadvantage of tissue factor activation that occurs when samples of Factor VII are obtained.

Before the present invention, however, there was not a Factor XIIa assay that was rapid, selective,

sufficiently sensitive to detect accurately XIIa at levels below about 10ng/ml, and that could be automated readily for large scale use.

The method used previously to determine Factor XIIa is an enzyme assay in which a chromogenic substrate is hydrolysed. As well as the lack of sensitivity mentioned above, a further disadvantage with this assay is that the chromogenic substrate is hydrolysed by a number of other substances that occur in plasma, including Factor Xa, kallikrein and thrombin. It is necessary, therefore, to estimate and make allowances for this hydrolysis, which will inevitably introduce inaccuracies, particularly at low XIIa levels. This assay cannot be regarded as giving accurate results at levels of XIIa below about 10ng/ml.

Approaches to improving the assay of XIIa and other clotting factors have involved the provision of improved chromogenic substrates see, for example, EP 78764-B and EP 285000-A.

Other, non-chromogenic types of assay have been proposed for blood clotting factors, for example, WO-8606489-A discloses the use of surface-bound fibrinogen and labelled fibrinogen. Immunoassays have also been proposed, for example, EP-325723-A discloses in general terms the use of a microparticle carrier sensitised with a monoclonal antibody to a blood clotting factor.

J62065693-A discloses a monoclonal anti-human blood coagulation Factor XI antibody. This antibody is stated

to have strong affinity to the active type factor XI as well as to the blood coagulation factor XI itself. The monoclonal antibody may be used for the determination of human blood coagulation Factor XI and the active type of Factor XI by various forms of immunoassay.

There has been described a monoclonal antibody that can recognise Factor XII and Factor αXIIa, but that does not recognise Factor βXIIa (E.J. Small et al, Blood, 65, 202-210, 1985). This is not surprising, because the authors found that the antibody was directed to the 40-Kd fragment that is released from Factor αXIIa when it is converted into Factor βXIIa, that is to say, the antibody was directed to an antigenic determinant that is part of the Factor XII and Factor αXIIa molecules but which physically does not exist in the βXIIa molecule.

The present invention provides a monoclonal antibody that binds to Factor βXIIa and that shows substantially no binding to Factor XII. A monoclonal antibody of the invention may bind to Factor βXIIa specifically or may also bind to Factor αXIIa.

It is surprising that, in contrast to J6206593-A and Small et al, the monoclonal antibodies of the present invention are able to recognise activated Factor XII and to discriminate between activated XII and the zymogen Factor XII itself.

The present invention provides a method of detecting and/or determining Factor XIIa or βXIIa in a sample,

which comprises subjecting the sample to a qualitative or quantitative immunoassay which comprises the interaction between an antigen and an antibody and the detection and/or determination of any resulting antibody-antigen complex, characterised in that the antibody is a monoclonal antibody of the present invention. Factor βXIIa is generally used as the standard for such an assay.

The present invention also provides a method of carrying out an immunoassay for an antigen in a sample of fluid, which assay comprises an interaction between the antigen and an antibody that binds thereto, and determining the amount of antigen present in the sample by reference to results obtained using pre-determined amounts of a known antigen, characterised in that the antibody is a monoclonal antibody of the invention and the known antigen is Factor βXIIa.

An immunoassay of the invention provides a rapid method of determination that can readily be used on automated equipment for large scale use. The assay is also accurate and sensitive, and can be used to detect levels of XIIa and βXIIa well below the effective lower limit of 10ng/ml for the previously used chromogenic assay.

The antibodies and immunoassays of the present invention are, accordingly, useful for assaying Factor XIIa or βXIIa, particularly when a large number of assays

are to be carried out, either in a research laboratory or in a clinical laboratory. An antibody and an immunoassay of the invention are particularly suitable for use in epidemiological studies to provide data that can be used in analogy to the Factor VII data discussed above in an assessment of the risk of heart disease, in particular, ischaemic heart disease and/or of acute myocardial infarction in an individual.

The present invention accordingly provides a method wherein an immunoassay of the invention is carried out on a sample of plasma obtained from a human subject and wherein, to determine the susceptibility of the subject to heart disease, the result obtained for the level of the antigen in the sample assayed is compared with the results obtained in a large-scale investigation which correlates the level of that antigen with susceptibility to heart disease. The individual assays and large-scale investigation preferably carried out using the same antibody. It is generally preferable also to use the same immunoassay.

Monoclonal antibodies and immunoassays of the present invention may also be used in studies of coagulation systems and of thrombotic disorders.

If it is desired to know whether a particular monoclonal antibody is βXIIa-specific or if it also binds to αXIIa, this may be done by carrying out an immunoassay on the antibody using Factor αXIIa as an antigen. The

immunoassay may be qualitative or quantitative, as desired, but it is generally preferable to use a liquid phase assay rather than a solid phase assay. In many cases, however, it is considered not to be essential to determine specificity towards αXIIa, since it is believed that satisfactory results will be obtained provided that the same antibody is used for all assays that will be compared with each other. (It will be noted that generally the results of an assay of the invention will be determined relative to βXIIa standards, and so may be considered as representing measurement of βXIIa and βXIIa-equivalents.)

The present invention also provides a peptide that is a fragment of βXIIa that is or that includes at least one antigenic determinant capable of recognising anti-Factor βXIIa. An antigenic fragment of βXIIa may itself be immunogenic or may be too small to be immunogenic, in which case it may be converted into an immunogen, for example, by conjugation to another peptide, for example, as described below. The term "antigenic fragment of βXIIa" as used herein includes both a peptide as defined above and an immunogenic form of such a peptide if it is not itself immunogenic.

Methods of preparing immunogens are known to those in the art. Any of these methods may be utilised to render immunogenic or to improve the immunogenicity of Factor βXIIa or antigenic fragment thereof.

In essence, all the methods comprise attaching a molecule which may not be antigenic or sufficiently antigenic to a larger protein molecule in order that an immune response or an improved immune response may be obtained on immunisation. Protein molecules used as carriers, are, for example, keyhole limpet haemocyanin, bovine serum albumin, bovine thyroglobulin and a purified protein derivative of tuberculin. For example, keyhole limpet haemocyanin (KLH) may be coupled to a cysteine-containing peptide via the cysteine thiol group of the peptide using a bifunctional cross-linking agent, for example, a maleimide reagent, for example, sulfosuccinimidyl-N-maleimido methyl cyclohexane-1-carboxylate, (see E. Isikawa et al J. Immunoassay 1983, 4, 209). The resultant conjugate may be purified by gel filtration chromatography and lyophilised.

A peptide that does not have an intrinsic thiol group may have a cysteine residue introduced at the N and/or C-terminus. A conjugate may be used to coat a solid phase, for example, wells of a plastics microtitre plate.

Factor βXIIa may be produced by a method which comprises first isolating Factor XII from fresh or freshly frozen plasma, for example, using a combination of ammonium sulphate precipitation and anion exchange chromatography for example, according to the method described by K. Fujikawa and E. W. Davie (Methods in

Enzymol, 1981, 80, 198-211). Methods for converting Factor XII to Factor βXIIa and isolating Factor βXIIa from the resulting mixture are described by K. Fujikawa and B. A. McMullen (Journal of Biol.Chem., 1983, 258, 10924-10933) and B. A. McMullen and K. Fujidawa (Journal of Biol. Chem. 1985, 260, 5328). To obtain Factor βXIIa, Factor XII is then generally subjected to limited cleavage, for example, by chemical or enzymatic digestion, for example, using trypsin or a trypsin-like enzyme, generally in a highly diluted form, for example, in a molar ratio of trypsin:Factor XII of 1:500, for example, in a weight ratio trypsin:Factor XII of 1:75 and the cleavage products separated, generally by chromatography.

Some preparations of Factor XII contain a substantial amount of αXIIa, as judged by examination of a reduced sample on SDS-PAGE, when three protein bands with apparent molecular weights of 80, 52 and 28-Kd are observed. Such Factor XII preparations also display amidolytic activity. According to Fujikawa & Davie, Factor XII and αXIIa can be separated using benzamidine-agarose column chromatography. On elution, two peaks are observed, both of which have clotting activity. However, only the second peak has amidolytic activity. Provided that the material applied to the benzamidine-agarose column contained no βXIIa, which may be determined by analysis of a non-reduced SDS-PAGE gel, then the second

peak is αXIIa. This can be confirmed by running reduced and non-reduced samples on SDS-PAGE.

An antigenic fragment of βXIIa may be produced by degradation of βXIIa by enzymatic or chemical means. For example the disulphide-linked light chain peptide of βXIIa can be obtained by reduction and carboxymethylation of βXIIa and isolation of the fragment by chromatography (K. Fujikawa and B. A. McMullen Journal of Biol. Chem. 1983, 258, 10924).

Alternatively, an antigenic fragment of βXIIa, if its amino acid sequence is known, may be produced synthetically, as may βXIIa itself. Any of the many known chemical methods of peptide synthesis may be used, especially those utilising automated apparatus.

An antigenic fragment according to the invention may be produced using the techniques of recombinant DNA technology, as may βXIIa itself. (Cool et al, 1985 and 1987, loc. cit. have characterised a human blood coagulation factor XII cDNA and gene.) This may be achieved, for example, by construction of a gene, for example, by chemical synthesis or by reverse transcription from the corresponding m-RNA, insertion of the gene into an appropriate vector, for example, a plasmid, for example, pBR 322, insertion of the vector into a host organism, for example, E. Coli, and expression of the gene in the host organism. Such procedures are now routine, particularly as vectors, for

example, PBR 322 are available commercially. Maniatis et al -, Molecular Cloning, Cold Spring Harbor Laboratory, 1982 is a standard work giving details of techniques used in this field. Generally chemical synthesis is preferred for smaller peptides, with recombinant DNA becoming more commercially attractive than chemical synthesis for large peptides.

Unless specified otherwise, the terms "Factor βXIIa" and "βXIIa" as used herein include antigenic fragments of the βXIIa molecule.

Factor βXIIa may be used to produce monoclonal antibodies or polyclonal antisera and these antibodies and antisera are part of the present invention. As indicated above, a monoclonal antibody of the present invention may be capable of binding to Factor βXIIa (it may be capable of recognising an antigenic determinant characteristic of βXIIa) and of showing no significant binding to Factor XII (capable of distinguishing between Factor βXIIa and Factor XII), or may be capable of binding to βXIIa and αXIIa, in which case the antibody may recognise antigenic determinants common to Factors βXIIa and αXIIa. Polyclonal antisera of the invention bind preferentially to βXIIa.

The term "antibody" as used herein includes any antibody fragment that is capable of binding antigen, for example, Fab and $F(ab')_2$ fragments.

As indicated above, a monoclonal antibody of the

present invention shows substantially no binding to Factor XII. For use in immunoassays for diagnostic purposes, the corrected cross-reactivity with Factor XII (see below) should generally be 0.1% or less. For other purposes, for example, for use as an immunoadsorbent, a higher cross-reactivity may be acceptable. A monoclonal antibody of the invention preferably has an affinity for Factor βXIIa of at least $10^{10}M^{-1}$.

A factor to take into consideration in assessing the cross-reactivity of an antibody of the invention with Factor XII is that even "pure" Factor XII preparations are almost inevitably contaminated with small amounts of XIIa (Silverberg and Kaplan, Blood 60, 1982, 64-70). The small amounts of XIIa present are not significant in most circumstances, but when assessing the degree of cross-reaction it is necessary to determine as accurately as possible the amount of XIIa in a Factor XII and then to take this into account to determine the corrected cross-reaction rather than the initially measured, apparent cross reaction. It has been found, for example, that Factor XII preparations may contain in the region of 0.5 to 0.8% XIIa. Taking this value into account, an apparent cross-reaction with Factor XII of 0.5% becomes a corrected cross reaction of less than 0.1%. Unless specified otherwise, the term "cross reaction" is used herein to mean the corrected cross reaction.

The present invention provides a method of producing

a monoclonal antibody of the invention, which comprises cultivating a hybridoma cell line capable of producing the antibody in a growth medium and obtaining the antibody from the growth medium.

The present invention further provides a method of producing a hybridoma cell line which produces a monoclonal antibody of the invention, which comprises administering an antigen to an animal to obtain antibody-producing cells, fusing the resulting antibody-producing cells with myeloma cells, and screening the resulting hybridomas for the production of the monoclonal antibody, wherein the antigen is Factor βXIIa or an antigenic fragment thereof.

Methods used to produce monoclonal antibodies are well known, see for example, Methods in Enzymology, H. Van Vunakis and J. J. Longone (Eds) 1981, 72(B) and ibid, 1983 92(E).

Monoclonal antibodies may be produced, for example, by a modification of the method of Kohler and Milstein (G. Kohler and C. Milstein, Nature, 1975, 256, 495): female Balb/C or C57/B10 mice are immunised by intraperitoneal injection of βXIIa or an antigenic fragment of βXIIa, for example, from 10 to 30 μg, generally 20 μg of βXIIa or a corresponding amount of the other antigen. The βXIIa or other antigen is preferably conjugated to another protein molecule, for example, to bovine thyroglobulin or to a purified protein derivative

of tuberculin. The conjugation may be carried out, for example, by a carbodiimide method or by using a hetero-bifunctional reagent. The immunogen is generally presented in an adjuvant, preferably complete Freunds adjuvant. This procedure is generally repeated at intervals, generally using the same immunogen in the same dose, for example, at 3 week intervals the mice are boosted with 20 μg of conjugated βXIIa in complete Freunds adjuvant until suitable response levels are observed. A pre-fusion boost is preferably given prior to sacrifice, for example, intravenously 3 days prior to sacrifice. The antibody response is monitored, for example, by RIA antisera curve analysis using ^{125}I-radiolabelled βXIIa or other βXIIa antigen prepared by the chloramine-T method (P. J. McConahey and F. J. Dixon, Int. Arch. Allergy Appl. Immunol, 1966, 29, 185). Purity is confirmed, for example, by using autoradiography, for example, of SDS-PAGE gels run under reducing conditions.

Immune mouse spleen cells are then fused with myeloma cells, for example, NSO mouse myeloma cells, for example in the presence of 40-50% PEG 4,000 or 50% PEG 1500. The cells are then seeded in wells of culture plates and grown on a selective medium. The supernatants are tested for reactivity against purified βXIIa or other βXIIa antigen, for example, by a solid phase enzyme immunoassay, for example, using peroxidase-labelled anti-mouse IgG. All wells showing specificity for βXIIa are

generally taken for further secondary screening. The secondary screening consists, for example, of screening all specific antibodies for binding in solution to βXIIa or to a βXIIa antigenic fragment that has been radiolabelled. These are preferably titrated to determine the antibody dilution required for 50% B max. Dose-response curves against cold, that is to say non-labelled, βXIIa or the corresponding cold antigenic fragment, and also against Factor XII, plasmin and fibronectin are preferably generated. The extent of cross reaction may be determined according to the following formula I:

$$\frac{\text{Weight of Cold Standard } \beta\text{XIIa to Achieve 50\% B max}}{\text{Weight of Cross-Reactant to achieve 50\% B max}} \times 100$$

(If an antigenic fragment has been used instead of βXIIa, this should be substituted in the above formula.)

Those antibodies showing a pre-determined apparent cross reactivity to factor XII, preferably of 1.5% or less, and more preferably 1% or less are taken forward. Scatchard analysis may be done on the dose-response data to produce values for the affinity constants for each antibody. Those having affinity constants of at least $10^{10}M^{-1}$ are generally taken forward for cloning. Successful clones are generally isotyped. The cells are then preferably sub-cloned by limiting dilution and again

screened, generally using an enzyme immunoassay, for the production of antibodies to βXIIa. A selected sub-clone from each cloning may also be evaluated with respect to specificity and dose response using a radioammunoassay.

A further screening process, using αXIIa as antigen, may be incorporated at a suitable point if, for particular purpose, it is desired to select clones that produce a monoclonal antibody that is shown to bind to both αXIIa and βXIIa or to βXIIa only, or to confirm the binding characteristics of an antibody produced by a chosen clone. For such screening there may be used an αXIIa preparation obtained according to the method of Fujikawa & Davie (see above). If, however, αXIIa screening is carried out after βXIIa screening it is not generally necessary to subject an αXIIa-containing Factor XII preparation to the benzamidine-agarose chromatoraphy described. It is generally sufficient to establish that the preparation is free from βXIIa. As indicated above, it is generally preferable to use a liquid phase assay rather than a solid phase assay, for example, an assay that involves the displacement by αXIIa of radiolabelled βXIIa from the antibody.

Sub-cloned hybridoma cells may be injected intra-peritoneally into Balb/C mice for the production of ascitic fluid. The immunoglobulin may be precipitated from ascites fluid, for example, at 4°C using saturated ammonium sulphate solution (equal volume). The

precipitate is preferably purified, for example, it may be centrifuged, dissolved, for example, in 50mM Tris-HCl buffer pH 7.5 (volume equal to original ascites volume) and then dialysed against the same buffer. The immunoglobulin fraction may then be further purified by anion exchange chromatography, for example, the protein solution may be applied to a Mono-Q anion exchange column (Pharmacia) and eluted using a salt gradient in the same buffer according to the manufacturer's recommendations. The fractions containing immunoglobulin are generally pooled and frozen at -20°C for storage.

Alternatively, hybridoma cells may be grown in culture for antibody production and the antibody isolated essentially as described above for ascites fluid.

Although the hybridomas described herein are derived from mouse spleen cells, the invention is not limited to hybridomas of murine or part-murine origin. Both fusion partners (spleen cells and myelomas) may be obtained from any suitable animal.

The present invention may involve the use of a polyclonal antiserum that binds to βXIIa.

Methods used to produce polyclonal antibodies are well known see, for example, "Practice and Theory of Enzyme Immunoassays", P. Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology, R. H. Burden and P. H. Van Knippenberg (Eds), Elsevier, 1985 and Introduction to Radioimunoassay and Related Techniques,

T. Chard, ibid , 3rd Edition, 1987. Polyclonal antisera may be produced, for example, in sheep or rabbit using Factor βXIIa, for example, conjugated to another protein, or Factor XII, as antigen.

As indicated above, the present invention also provides a method of detecting and/or determining Factor XIIa or βXIIa in a sample, which comprises subjecting the sample to a qualitative or quantitative immunoassay that comprises an interaction between an antigen and an antibody and the detection and/or determination of any resulting antibody-antigen complex, characterised in that the antibody is a monoclonal antibody of the present invention.

The present invention further provides a method of carrying out an immunoassay for an antigen in a sample of fluid, which assay involves an interaction between the antigen and an antibody that binds thereto and also involves determining the amount of antigen present in the sample, by reference to results obtained using pre-determined amounts of a known antigen, characterised in that the antibody is a monoclonal antibody of the invention and the known antigen is Factor βXIIa.

Methods of carrying out immunoassays are well known cf for example, Methods in Enzymology, H. Van Vunakis and J. J. Langone (Eds), 1981, 72(B); Practice and Theory of Enzyme Immunoassays, P Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology, R. J. Burden and P.

H. Van Knippenberg (Eds), Elsevier, 1985; Introduction to Radioimmunoassay and Related Techniques, T. Chard, ibid, 3rd Edition, 1987; and Methods in Enzymology, H. Van Vunakis and J. J. Langone (Eds) 1981, 74(C).

As indicated above, immunoassay techniques, both qualitative and quantitative, are well known and include ELISA (enzyme linked immunosorbent assays), Western blotting, fluid phase precipitation assays, coated particle assays, competitive assays, sandwich assays, including forward, reverse and simultaneous sandwich assays, and solid phase radio immunoassays (SPRIA).

Of these, ELISA and SPRIA may be particularly convenient in the present case. Accordingly, samples of a monoclonal antibody according to the present invention may be adsorbed on to a solid phase support, for example, a plastics or other polymeric material, for example the wells of plastics microtitre plates, samples of plasma under investigation or a standard solution are incubated in contact with the antibody reagent and any resulting bound Factor XIIa or βXIIa detected using a labelled antibody that is capable of binding to the bound Factor or to the standard, for example, to a different epitope on the molecule. The labelled antibody may be polyclonal or monoclonal. Any appropriate radioisotope may be used as the label, for example a β-emitter or a α-emitter, examples being ^{125}I, ^{131}I, ^{3}H, and ^{14}C. An enzyme label is, for example, alkaline phosphatase, using

phenolphthalein monophosphate as substrate. An enzyme reaction may be followed using an electrochemical method. As an alternative to the use of a labelled anti-antibody, bound XIIa or βXIIa may be determined directly by using a chromogenic substrate.

Instead of adsorbing a monoclonal antibody of the invention on a solid support, βXIIa itself or an antigenic fragment thereof, may be bound for use in a competitive assay. A further alternative is to use labelled, for example, radiolabelled βXIIa or an antigenic fragment thereof in a competitive assay.

An example of a radioimmunoassay for use according to the invention is as follows: A dose-response curve using a monoclonal antibody ^{125}I-labelled βXIIa, βXIIa standard solutions and anti-mouse IgG coupled to a solid support, for example, Sephacryl S-1000 may be demonstrated according to the following method: Monoclonal antibody ascitic fluid is diluted with Assay Buffer, for example, a buffer comprising 50mM Tris-HCl pH 7.4 containing 0.15m NaCl, 0.25% BSA, 10mM EDTA, 3mM NaN and 0.1% Triton. In duplicate assay tubes is added an sample of each of a monoclonal antibody solution, a ^{125}I-radiolabelled βXIIa solution and purified βXIIa standard solution in Assay Buffer. The standard solutions are prepared from a βXIIa stock solution. Control tubes to give total counts were prepared using Assay Buffer and tracer solution. All tubes are mixed and then incubated.

After this a sample of a suspension containing an optimal amount of anti-mouse IgG coupled to a solid support was added to each tube (except totals) and the tubes are then incubated with shaking. After this step preferably sucrose buffer (Assay Buffer + 10% w/w sucrose) is layered underneath the reaction mixture in each tube (except totals), for example, using a peristaltic pump. The solid support-coupled anti-mouse IgG is allowed to sediment after which the liquid is removed from each tube. All tubes including totals are then counted. The % bound may be calculated by dividing the counts achieved for each βXIIa standard by the total counts. The total counts added are, for example, 10,000 cpm. In the above radioimmunoassay an antigenic fragment of βXIIa may be substituted for βXIIa.

The present invention further provides a kit for carrying out an immunoassay of the present invention, which kit comprises, each in a separate container or otherwise compartmentalised:
a monoclonal antibody of the invention and Factor βXIIa or an antigenic fragment thereof. The kit may comprise further components for carrying out an immunoassay, for example, as described above. The monoclonal antibody of the invention may be unlabelled or labelled. It may be immobilised on a solid support.

A kit according to the invention may comprise, for example,

a)(i) a monoclonal antibody of the present invention, or (ii) Factor βXIIa or an antigenic fragment thereof, or (iii) an antibody directed against an antibody of the invention;

b)(i) labelled antibody capable of reacting directly or indirectly with Factor βXIIa, or

(ii) labelled Factor βXIIa, or

(iii) a chromogenic substrate for Factor βXIIa; and

c) a control reagent, which is purified Factor βXIIa or an antigenic fragment thereof.

A component a)(i), a)(ii) or a)(iii) may be bound to a solid support, if desired.

A kit may also comprise further components, each in a separate container, for example, wash reagent solution and substrate solution.

Polyclonal antisera or, especially, a monoclonal antibody of the invention may be used in affinity chromatography as an immunosorbent in the purification of Factor βXIIa or an antigenic fragment thereof and the present invention provides an immunosorbent comprising polyclonal antisera or, especially, a monoclonal antibody of the present invention, generally absorbed on or otherwise carried by a solid support in a conventional manner (see below), and also provides a method of purifying Factor βXIIa or an antigenic fragment thereof using such a supported antibody (immunosorbent). The purified Factor βXIIa or antigenic fragment may be used

as the control reagent in the method of the present invention for determining Factor βXIIa. Conversely, the invention also provides an immunosorbent comprising Factor βXIIa or an antigenic fragment thereof, generally absorbed on or otherwise carried by a solid support in a conventional manner (see below).

The present invention also provides a method of screening and/or a method of purifying anti-Factor βXIIa antibodies and antisera using such a supported antigen (immunosorbent). It is advantageous to screen monoclonal antibodies for specificity and/or affinity for βXIIa using an antigenic fragment of βXIIa preferably in the form of an immunosorbent as described above. If desired, further screening and/or purification may be carried out using Factor αXIIa or an antigenic fragment thereof immobilised on a solid support as an immunosorbent.

Polyclonal antibodies may be purified, expecially to increase the content of anti-Factor βXIIa antibodies, for example, by contacting the polyclonal antibodies with Factor βXIIa or an antigenic fragment thereof. The polyclonal antibodies preferably are contacted with an antigenic fragment of βXIIa immobilised on a solid support, and the resulting bound antibodies are released. The specificity and affinity properties of a crude polyclonal antibody preparation can be improved substantially by such a method, so the resulting polyclonal antibodies are made more suitable for use in a

commercial assay.

A monoclonal mouse antibody that binds to βXIIa may be covalently coupled to CNBr-activated Sepharose-4B (Pharmacia) according to the manufacturer's instructions. Such a column may be used to isolate a βXIIa antigen, for example, to isolate βXIIa from plasma or βXIIa or an antigenic fragment thereof from a digest of Factor XII, as appropriate. Bound βXIIa antigen may be eluted from the column using, for example, 4M guanidine, and the antigen detected in the effluent, for example, βXIIa may be detected in the effluent fractions by enzymatic activity using S-2302 peptide substrate (Kabi) or using ^{125}I labelled βXIIa.

A βXIIa antigen as described above that is to say, βXIIa itself or an antigenic fragment thereof, may, for example, be coupled to thiol-activated Sepharose (Pharmacia) via one or more thiol groups on intrinsic or introduced cysteine moieties according to the manufacturer's instructions. Factor αXIIa or an antigenic fragment thereof may be coupled analogously.

As indicated above, there appears to be an enzyme/substrate relationship between Factor XIIa, for example Factor βXIIa, activity and Factor VII activity, which suggests that Factor XIIa, for example Factor βXIIa, activity can be used instead of Factor VII activity as an indicator of the risk of heart disease, particularly ischaemic heart disease (IHD) and acute

myocardial infarction (AMI).

Results of investigations into turnover of ^{125}I-labelled Factor XII in rabbits fed on normal or cholesterol-supplemented diets indicate that, although there is little change in the half-life and pool size of Factor XII in the extravascular and intravascular compartments, and although there is little change in the fractional catabolic rate, the absolute catabolic rate in the rabbits fed on a cholesterol-supplemented diet is greater than that in rabbits fed on the standard diet. Rabbits have much lower kallikrein levels that man, so it would be expected that an increase in catabolic rate of Factor XII would be even more marked in man in corresponding circumstances. The significance of the increased catabolic rate of Factor XII is the effect this will have on Factor VII activity. These results indicate that Factor XIIa, for example Factor βXIIa, may be suitable as an indicator of the risk of AMI.

Accordingly, it is proposed that data resulting from an immunoassay of the invention carried out on a sample of plasma obtained from a subject will be useful in indicating the risk to that subject of heart disease, in particular Ischaemic Heart Disease and/or Acute Myocardial Infarction. A correlation between the results obtained for an individual and the risk of heart disease, for example, ischaemic heart disease and especially acute myocardial infarction for that individual may be made by

studying these parameters in a number of individuals, preferably including individuals considered on the basis of other criteria to be at risk and individuals not considered to be at risk. The larger the population investigated the better will be the accuracy of the correlation. The design and conduct of such epidemiological studies is known, see, for example, the Northwick Park Hospital and Framingham Studies referred to above. Assessment of the risk to an individual of heart disease, for example AMI, may be made by comparison of the data determined for that individual with that determined in epidemiological studies, for example as described above.

The method and other embodiments of the present invention are also useful in investigations of the mechanisms involved in blood coagulation amd in the study of thrombotic disorders.

The following Examples illustrate the invention, but are not intended to limit the invention in any way.

EXAMPLE 1

Isolation and purification of Factors XII and βXIIa

Isolation of Factor XII from fresh or frozen human plasma was carried out using a combination of ammonium sulphate precipitation and anion exchange chromatography essentially as described by K. Fujikawa and E. W. Davies, Methods Enzymol, 1981, 80, 198). From 6.5 litres of

plasma the yield of factor XII was 53mg.

The preparation of Factor βXIIa from Factor XII by tryptic digestion was carried out using the method previously described by B. A. McMullen and K. Fujikawa JBC, 1985 260, 5328; and by K. Fujikawa and B. A. McMullen JBC, 1983, 258, 10924). (JBC is Journal of Biological Chemistry.)

The 53mg of Factor XII were dialysed against 8 litres of 50mM Tris/75mM NaCl, pH 8.0 at 4°C overnight. The protein solution was warmed to 37°C, 0.7mg trypsin was added and equilibrated at 37°C for 15 minutes. 1.4mg of soya bean trypsin inhibitor (SBTI) was then added to stop the digestion and the solution was kept at 37°C for a further 15 minutes. The βXIIa was separated immediately from the other components using DEAE-Sephacel. The column was washed with Tris/75mM NaCl pH 8.0 until some protein had washed off and then a gradient was started using 50mM Tris/75mM NaCl->50mM Tris/500mM NaCl pH 8.0. Two protein peaks were eluted during the gradient. βXIIa has no clotting activity, unlike Factor XII. The activity of the product in the eluate was measured using the synthetic substrate S-2302 (Kabivitrum) and the rate of change of absorbance at 405nm. βXIIa appears in the second peak. 14mg of βXIIa were obtained (70% from Factor XII).

Figure 1 of the accompanying drawings is a plot of absorbance at 280nm against time for the eluate of the

DEAE-Sephacel column and shows βXIIa in the second peak.

Purity was confirmed by SDS-PAGE under reducing and non-reducing conditions according to the method of Laemmli. (V. K. Laemmli, Nature, 1970, 227, 680). The acrylamide concentration was 3% in the spacer gel and 10% in the separating gel. Protein bands were detected by Coomassie blue staining. Figures 2a and 2b of the accompanying drawings show of SDS-PAGE gels for XII and βXIIa, respectively. $E_{280}^{1\%}$ =1.42 in Figure 2a and $E_{280}^{1\%}$ =1.52 in Figure 2b. Track 1 of both gels consists of bands formed by molecular weight standards as follows: 20,100; 24,000; 29,000; 36,000; 45,000; and 66,000. Tracks 1, 2 and 3 of Figure 2a show the bands obtained for three fractions from the Factor XII peak on S-Sepharose chromatography (which was used instead of the CM-cellulose chromatography described by Fujikawa). Tracks 2 to 7 of Figure 2b show the bands obtained for fractions 32 to 27 respectively of the eluate of the DEAE-Sephacel chromatography.

Example 2

(a) Production of murine specific monoclonal antibodies

Murine monoclonal antibodies to human βXIIa were prepared by a modification of the method of Kohler and Milstein (G. Kohler and C. Milstein, Nature, 1975, 256, 495).

Female Balb/C mice were immunised by intraperitoneal

injection of 20 μg of βXIIa conjugated to a) bovine thyroglobulin or b) a purified protein derivative of tuberculin. The conjugation was done by either a carbodimide method or using a heterobifunctional reagent. The immunogen was presented in complete Freunds adjuvant. At 3 week intervals the mice were boosted with 20 μg of conjugated βXIIa in complete Freunds adjuvant. A pre-fusion boost was given intravenously 3 days prior to sacrifice.

The antibody response was monitored by RIA antisera curve analysis using ^{125}I- radiolabelled βXIIa prepared by the chloramine-T method (P.J. McConahey and F.J. Dixon, Int. Arch. Allergy Appl. Immunol, 1966, 29, 185). Purity was confirmed using autoradiography of SDS-PAGE gels run under reducing conditions. Immune mouse spleen cells were fused with NSO mouse myeloma cells in the presence of 40-50% PEG 4,000. The cells were then seeded in wells of culture plates and grown on selective medium. The supernatants were tested for reactivity against purified βXIIa by a solid phase enzyme immunoassay using peroxidase-labelled anti-mouse IgG. Briefly, the wells of a 96 well microtitre plate were coated with purified βXIIa (100 μl of a 10 μg/ml solution in phosphate buffered saline, PBS) and then blocked using 2% BSA (bovine serum albumin) in PBS. The cell culture supernatants were added to the wells and after incubation for 1 hour at 37°C the wells were washed 3 times and

peroxidase-labelled anti-mouse IgG was added at an optimal dilution. After a further incubation at 37°C for 1 hour the wells were again washed and 100 µl of substrate slution was added (6mM H_2O_2 and 40 mM O-phenylenediamine in 0.1 M citrate pH 5.0). The colour development was stopped with 100 µl of 3M HCl and the absorbance was measured at 492nm.

All wells showing specificity for βXIIa were taken for further secondary screening. The secondary screening consisted of screening all specific antibodies for binding to radiolabelled βXIIa in solution. These were titrated to determine the antibody dilution required for 50% B max. Dose response curves against cold (non-labelled) βXIIa, factor XII, plasmin and fibronectin were generated. The extent of cross reaction was determined on the following formula:

$$\frac{\text{Weight of Cold Standard } \beta\text{XIIa to Achieve 50\% Bmax}}{\text{Weight of Cross-reactant to Achieve 50\% B max}} \times 100$$

Those antibodies showing an apparent cross reactivity to Factor XII of less than 1.5% were taken forward. Scatchard analysis was done on the dose-response data to produce values for the affinity constants for each antibody. Those having affinity constants of at least $107M^{-1}$, and preferably up to $10^{10}M^{-1}$ were taken forward for cloning. Successful clones were isotyped.

The cells were then sub-cloned by limiting dilution and again screened using enzyme immunoassay for the production of antibodies to βXIIa. A selected clone from each cloning was also evaluated using radioimmunoassay with respect to specificity and dose response. Sub-cloned hybridoma cells secreting antibody to βXIIa were injected intra-peritoneally into Balbc mice for the production of ascitic fluid. Six cloned hybridomas that produce ascites fluid containing monoclonal antibodies to βXIIa were obtained.

b) Isolation of Immunoglobulin Fraction from Ascites Fluid

The immunoglobulin fraction was precipitated from ascites fluid at 4°C using saturated ammonium sulphate solution (equal volume). The precipitate was centrifuged, dissolved in 50 mM Tris-HCl buffer pH 7.5 (volume equal to original ascites volume) and then dialysed against the same buffer. The protein solution was then applied to a Mono-Q anion exchange column (Pharmacia) and eluted using a salt gradient in the same buffer according to the manufacturer's recommendations. The fractions containing immunoglobulin were pooled and frozen at -210°C for storage. The yield was generally from 1 to 5 mg purified antibody/ml of ascitic fluid.

c) Enzyme Labelling of Antibodies

Purified immunoglobulin from ascitic fluid or polyclonal antisera was conjugated to alkaline phosphatase using the thiol-maleimide method (E. Ishikawa et al , J. Immunoassay, 1983 4, 209).% The conjugates obtained were purified by gel-filtration chromatography using Sephacryl S-300 (Pharmacia).

Example 3

Preparation of Affinity Support

Monoclonal mouse antibody to βXIIa was covalently coupled to CNBr activated Sepharose-4B (Pharmacia) according to the manufacturer's instructions. From 5 to 10 mg of purified IgG was bound to 1 g of unswollen gel. This column was used to isolate βXIIa from plasma of from tryptic digests of Factor XII. 4M guanidine was used to elute bound βXIIa from the column and βXIIa was detected in the effluent fractions by enzymatic activity using S-2302 peptide substrate (Kabi) or by using ^{125}I-labelled βXIIa.

Example 4

Polyclonal Antisera

Polyclonal antisera to factor XII and βXIIa were raised in sheep or rabbits by standard methods using native factor XII and conjugated βXIIa, see Methods in Enzymology, H. Van Vunatis and J. J. Langone (Eds) 1981, 72(B) and 1983 92(E).

Example 5

Radioimmunoassay for βXIIa

A dose-response curve using monoclonal antibody 202/2.6 obtained according to the method described in Example 2. ^{125}I-labelled βXIIa, βXIIa standard solutions and sheep anti-mouse IgG coupled to Sephacryl S-1000 (SAM-Sephacryl) was demonstrated accordingly to the following method: Monoclonal antibody 202/2.6 ascitic fluid was diluted 1:1000 with Assay Buffer (50mM Tris-HCl pH 7.4 containing 0.15M NaCl, 0.25% BSA, 10mM EDTA 3mM NaN3 and 0.1% Triton). In duplicate 4 ml volume polystyrene assay tubes was added: 50 µl of monoclonal antibody solution, 50 µl of radiolabelled βXIIa solution and 100 µl of pure βXIIa standard solution in Assay Buffer. The standard solutions were prepared by doubling dilutions of a βXIIa stock solution. The concentration of the stock solution was calculated using the following formula:

E^{1}_{280} = 15.2 (K. Fujikawa and B.A. McMullen JBC, 280 1983, 258, 10924).

Control tubes to give total counts contained 150 µl of Assay Buffer and 50 µl of tracer solution. All tubes were Vortex mixed and then incubated at 21±1°C for 19 hours. After this period 50 µl of a suspension containing an optimal amount of SAM-Sephacryl was added to each tube (except totals) and the tubes were then incubated at

21±1°C for 1 hour with shaking. After this step 1.5 ml of sucrose buffer (Assay Buffer + 10% w/w sucrose) was layered underneath the reaction mixture in each tube (except totals) using a peristaltic pump. The SAM-Sephacryl was allowed to sediment at 21±1°C for 30 minutes after which the liquid was removed from each tube, leaving approximately 0.3 ml. All tubes including totals were then counted for 60 seconds in a Multiwell gamma counter. The results are presented in Table 1 and Figure 3 of the accompanying drawings, which is a dose-response curve of βXIIa concentration against % bound ^{125}I βXIIa. The % bound was calculated by dividing the counts achieved for each βXIIa standard by the total counts. The total counts added were 10,000 cpm.

TABLE 1

Concentration of Cold βXIIa added (μg/ml)	% Bound 1	2	Mean % Bound
90	2.1	1.8	2.0
45	2.3	2.4	2.4
22.5	2.6	3.0	2.8
11.3	3.4	3.2	3.3
5.6	4.6	4.0	4.3
2.81	5.8	6.2	6.0
1.41	9.3	9.6	9.4
0.73	13.6	12.8	13.2
0.35	19.0	19.5	19.2
0.18	24.0	25.0	24.5
0.09	26.7	26.7	26.7
0.05	29.8	27.9	28.8
0.02	27.7	28.4	28.0
0.01	29.2	29.4	29.3
0.005	28.7	28.9	28.8
0.00	29.0	30.2	29.6

EXAMPLE 6

Production of βXIIa monoclonal antibodies

Murine monoclonal antibodies to human βXIIa were prepared by the general method of Kohler and Milstein (G. Kohler & C. Milstein, Nature, 1975, 256, 495):

(i) Preparation of the immunogen

The immunogen was prepared by conjugating βXIIa to a purified protein derivative of tuberculin (PPD) using the heterobifunctional reagent sulpho SMCC (P.J. Lachmann et al, Synthetic Peptides as Antigens, Ciba Foundation Symposium 119, 25-57, 1986):

N-Succinidimyl 3-(2-pyridyldithio)propionate (SPDP, 6mg) was dissolved in ethanol (5ml). A 5mg/ml solution of PPD (for Heaf Test, Statens Serum Institut, Denmark) in 0.1M PBS pH 7.4 was prepared. A mixture of PPD solution (1ml) and SPDP solution (5μl) was incubated for 30 minutes at room temperature, after which the preparation was dialysed against 0.1M PBS pH 7.4. Free thiol groups were exposed by incubating the derivatised PPD with dithiothreitol (at a 50mM concentration) for 30 minutes at room temperature. The reaction mixture was then dialysed against 0.1M sodium acetate buffer pH 4.5 containing 100mM sodium chloride.

Purified βXIIa, prepared as described in Example 1

above, was dialysed against 0.1M borate buffer pH 8.0, and the final concentration was adjusted to 5mg/ml. A solution of sulpho-succinimidyl 4-(N-maleiimidomethyl) cyclohexane-1-carboxylate (sulpho-SMCC) was prepared in 0.1M borate buffer pH 8.0. 100µl of this solution was added to the βXIIa solution such that the molar ratio sulpho-SMCC:βXIIa was 100:1. The mixture was incubated at room temperature for 30 minutes and then dialysed against 0.1M PBS pH 7.4.

Equal weights of activated βXIIa and activated PPD were incubated for 18 hours at 4°C and the mixture was then dialysed against 0.1M PBS pH 7.4. This material was used for immunisation.

(ii) Production of spleen lymphocytes

Female C57/B10 or Balb/C mice were primed with BCG, and one day later were immunised with 20µg of the βXIIa-PPD immunogen in complete Freund's adjuvant. At two week intervals, the mice were boosted with 20µg of the immunogen in incomplete Freund's adjuvant. Two weeks after the second boost, a pre-fusion boost was given intravenously, and three days later the animals were sacrificed.

The immune response of the mice was monitored by RIA using ^{125}I-radiolabelled βXIIa prepared using the chloramine-T method with an uptake of approximately 50-75 µCi/mg. Purity was confirmed using autoradiography of

SDS-PAGE gels run under reducing conditions.

The RIA procedure was as follows: To 100μl of diluted labelled βXIIa (15,000 cpm/tube) was added 100μl of antiserum diluted in assay buffer II (phosphate buffered saline containing 0.5% (w/v) Tween (Trade Mark), 1% (w/v) bovine serum albumin (BSA) and 0.01% (w/v) sodium azide). A further 100ul of buffer was added and the mixture was incubated at 20°C for 20 hours. The bound labelled βXIIa was separated using a second antibody system (Dako anti-mouse antibody at 1/100 in assay buffer II containing 6% (w/v) PEG and 50μg of 1/100 normal mouse serum). After decanting off, the supernatant was read in a gamma counter, 60s/tube.

(iii) Fusion Protocol

Spleen cells were removed from responding mice having an antibody titre greater than 1/5,000 and preferably greater than 1/20,000, gently homogenised, washed three times and then resuspended in Dulbecco's modified Eagle's medium (DMEM). The myeloma cell line used was NSO (uncloned) obtained from the MRC Laboratory of Molecular Biology, Cambridge. The myeloma cells, in log growth phase, were washed in DMEM.

Spleen cells ($1x10^8$) were mixed with myeloma cells ($7x10^7$), centrifuged, and the liquid removed. The resultant cell pellet was placed in a vessel in a 37°C water bath. Over a period of one minute, 1ml of a 50%

(w/v) solution of polyethylene glycol (PEG) 1500 in saline Hepes pH 7.5 was added and the mixture stirred gently for one and a half minutes. Over a period of five minutes 50ml of serum-free DMEM was added, then the mixture was centrifuged. The supernatant was discarded and the cell pellet resuspended on 10ml of DMEM containing 18% foetal calf serum (FCS). A 10μl aliquot of the resulting cell suspension was added to each of the 480 wells of standard multiwell tissue culture plates. Each well contained 2ml of standard HAT medium (hypoxanthine, aminopterin, thymidine) and a feeder layer of Balb/C cells at a concentration of $5x10^4$ macrophages/well. The wells were maintained at 37°C in 9% CO_2 air at approximately 90% humidity. The wells were analysed for monoclonal antibody production as described below. From those wells that yielded antibody-producing cells, cells were removed and cloned by the standard limiting dilution cloning procedure.

(iv) Screening of hybridomas using solid phase enzyme immunoassay

All wells showing hybrids were screened using a solid phase enzyme immunoassay (EIA) as follows:

The wells of a 96 well microtitre plate (Nunc Immunoplate, Polysorb Catalogue No. 4-75094) were coated overnight with purified βXIIa obtained as described in Example 1 above, using 100μl aliquots of a 1μg/ml

solution in MES-saline (20mM 2-(N-morpholinoethane sulphonic acid), 150mM sodium chloride, pH6.5) and then blocked using 1% milk protein in MES-saline. The cell culture supernatants diluted 1:1 in augmented MES-saline (MES-saline containing 0.05% (w/v) Tween, 1% (w/v) BSA and 0.05% (w/v) Thiomersal) were added to the wells and after incubation for 1 hour at 37°C the wells were washed three time with PBS containing 0.05% (w/v) Tween and 0.05% (w/v) Thiomersal, and horse radish peroxidase-labelled anti-mouse IgG (Biorad, anti-heavy chain specific, 1:2500 in augmented MES-saline was added. After a further incubation at 37°C for 1 hour the wells were again washed and 100µl of substrate solution added (6mMH_2O_2 and 40mMo-phenylenediamine in 0.1M citrate, pH 5.0). The colour development was stopped with 100µl of 3M HCl, and the absorbance was measured at 492nm. Wells showing a good discrimination between the binding of βXIIa and Factor XII were titrated in an antisera curve analysis versus ^{125}I-labelled βXIIa. All wells showing binding of the labelled βXIIa and titres greater than 1 in 10 were presented for analysis of the dose response and of the extent of cross reaction with Factor XII using formula I:

$$\frac{\text{Weight of Cold Standard } \beta\text{XIIa to Achieve 50\% Bmax}}{\text{Weight of Cross-Reactant to Achieve 50\% Bmax}} \times 100$$

From this preliminary data 6 hybridomas having an apparent cross reactivity of 1.5% were selected for cloning.

A representative clone from each line was further analysed by RIA for cross-reaction with Factor XII (FXII), plasmin and fibronectin. Table 2 gives the dose response curves for three preferred clones to determine the apparent cross reactivity to Factor XII.

TABLE 2

RIA Dose Response To Determine Apparent Cross Reactivity Of Certain Clones to Factor XII

	Clone Number		
Concentration of βXIIa ng/ml	201/9	2/215	2/15
	%Bound		
0.0	100	100	100
0.5	95	87	87
1.0	91	87	79
2.5	76	56	57
5.0	58	33	40
10.0	32	17	21
50.0	6	3	4
100.0	4	2	2
Concentration of XII ng/ml			
50	97	98	83
100	94	87	73
500	71	37	26
1000	51	19	13
2000	30	9	7

Scatchard analysis was performed on the dose response curves to give affinity constant (Ka) values. The data for three preferred cell lines is given in Table 3 below, together with some data relating to a further clone having a high degree of cross-reactivity to Factor XII:

TABLE 3

RIA Dose Response and Apparent Cross-Reactivity Study of Certain anti-βXIIa antibodies

Clone Number	Titre	Bo/T%	βXIIa ng/ml	Cross Reactivity FXII	Plasmin	Fibronectin	Ka (M^{-1})
201/9	200,000	30	6.5	0.7	< 0.1	< 0.1	3.5×10^{10}
2/15	20,000	25	3.2	1.4	< 0.1	< 0.1	6.5×10^{10}
2/215	20,000	12	3.1	1	< 0.1	< 0.1	1×10^{10}
202/16.1.9	5,000	-	2,000	67	-	-	5.46×10^{10}

(v) Assessment of Corrected Cross-Reactivity with Factor XII

A factor to be taken into consideration in assessing the cross-reactivity of an anti-βXIIa antibody with Factor XII is that even "pure" Factor XII is almost inevitably contaminated with small amounts of XIIa (Silverberg and Kaplan, Blood 60, 1982, 64-70). Although

this contamination is not significant for most purposes, the presence of even small amounts of XIIa will clearly affect the results of studies of cross-reactivity of anti-βXIIa antibodies with Factor XII. Accordingly, it was considered necessary to obtain an estimate of the level of βXIIa in the samples of Factor XII used for the cross-reactivity determinations. The chromogenic assay for XIIa, which can detect contamination at levels >10ng/ml was used.

Measurement of XIIa concentration in Factor XII sample

The amidolytic activity of XIIa present in the "pure" factor XII was determined by the addition of 200µl of 2mM Pro-Phe-Arg-p-nitroanilide (S2302, Kabi) in 65mM Tris, 135mM sodium chloride, 0.01% BSA, pH 8 to 50µl of βXIIa standards or of the Factor XII samples, which have predetermined concentrations of XII (determined by measurement of the extinction co-efficient, see Fujikawa & McMullen, loc. cit.). The hydrolysis of the substrate was measured by determination of the absorbance at 405nm after incubation at room temperature for 60 minutes. The amidolytic activity of the samples was compared with that of the βXIIa standards. The results obtained are presented in Table 4 below.

TABLE 4

Amidolytic activity of Factors βXIIa and XII

Sample or Standard Concentration	Absorbance at 405 nm	βXIIa (ng/ml)
βXIIA STANDARDS		
0	0.06	
10	0.10	
25	0.15	
50	0.25	
100	0.41	
FACTOR XII SAMPLES		
1200	0.09	<10
12000	0.30	60
24000	0.50	>100

The percentage contamination of factor XII with βXIIa was calculated according to the following formula:

$$\frac{\text{Concentration of } \beta\text{XIIa in factor XII}}{\text{Concentration of factor XII}} \times 100$$

The results obtained indicate that the contamination of factor XII by XIIa is within the range of from 0.5 to 0.8%.

Assessment of cross-reactivity of factor XII with monoclonal antibody 2/215

An immunoassay for βXIIa was carried out using monoclonal antibody conjugates 201/9 and 202/16.1.9 as described in Example 7 below. The βXIIa standards and Factor XII sample concentrations used were as set out for the chromogenic test above. The results are shown in Table 5

TABLE 5

Cross Reactivity of 2/215 Using Monoclonal Antibody Conjugates

Concentration of βXIIa ng/ml	Absorbance at 550 nm
0.0	0.114
1.0	0.213
2.5	0.354
5.0	0.587
10.0	0.999
15.0	1.405
25.0	1.960

Concentration of Factor XII µg/ml	Absorbance at 550 nm
0.23	0.266
0.46	0.322
1.20	0.652
2.30	1.065
5.80	1.905

The apparent cross-reactivity was calculated to be in the region of 0.5%, but when contamination of the Factor XII with XIIa was taken into account as described above, the corrected cross-reactivity was found to be less than 0.1%.

(vi) Production of antibody

Sub-cloned hybridoma cells that secrete antibody to βXIIa were injected intra-peritoneally into Balb/C mice for the production of ascitic fluid or were grown in culture.

The immunoglobulin fraction was precipitated from ascites fluid at 4°C using an equal volume of saturated ammonium sulphate solution. The precipitate was centrifuged, dissolved in a volume of 20mM Tris-HCl buffer pH 7.5 (Buffer A) equal to the original volume of the ascites fluid, and then dialysed against the same buffer. The protein solution was then fractionated on a Mono-Q (Trade Mark) anion exchange column (HR 10/10, Pharmacia) by means of FPLC (Trade Mark) equipment from Pharmacia. Elution was conducted using a gradient made with buffer A and buffer B (buffer A supplemented with 1M NaCl) at a flow rate of 2ml per minute. The eluate was monitored at 280nm and 1ml fractions were collected. The fractions containing immunoglobulin were pooled and frozen at -20°C for storage.

Essentially the same method was used to isolate the immunoglobulin fraction from culture fluid.

(vii) Deposit of hybridomas

Hybridoma cell lines have been deposited at the European Collection of Animal Cell Cultures, Division of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, England, as follows:

2/215 (BFx11a) deposited on 16 January 1990, deposit number 90011606;

201/9 (ESBT4 1.1) deposited on 18 January 1990, deposit number 90011893;

202/16.1.9 (ESBT 92.9) deposited on 25 January 1990, deposit number 90012512.

EXAMPLE 7

Assay using monoclonal antibody 2/215

The wells of 96 well microtitre plates were coated overnight at 20-25°C with antibody 2/215 using 100µl per well of a 5µg/ml preparation of the antibody in a coating buffer (0.1M phosphate buffer, 0.15M sodium chloride containing 0.1% sodium azide, 0.02% gentamycin sulphate, pH 7.4.

Aliquots of 100µl of a sample of human plasma or of βXIIa standards were added to the wells, and the plates were incubated at 20-25°C for 1 hour. Each well was then

washed with wash buffer (10mM borate buffer, 50mM sodium chloride, 0.1% Triton-X-100, 0.05% sodium azide, pH 7.4) prior to the addition of a mixture of anti-βXIIa monoclonal antibodies 201/9 and 202/16.1.9 (202/16.1.9 being a high Factor XII cross reacting antibody) which antibodies had previously been conjugated to alkaline phosphatase using the thiol-maleiimide method (E. Ishikawa et al, J. Immunoassay, 1983, 4, 209). The antibody conjugates were each titrated against βXIIa to determine the optimal dilution. The conjugates were then mixed at these dilutions. The conjugate diluent is 0.1M sodium chloride, 1mM magnesium chloride hexahydrate, 0.1M Tris, 0.1mM zinc chloride, 0.1% (w/v) sodium azide, 0.1% Triton-X-100 and 1% BSA. After incubation at 20-25°C for 1 hour, each well was again washed with the wash buffer.

The plate was blotted firmly on an absorbent pad prior to the addition of 100µl of phenolphthalein monophosphate substrate solution (1.0g/l phenolphthalein monophosphate (PMP) in 0.5M diethanolamine, pH8.6 containing 0.02% Bronidox). The plates were incubated at 20-25°C for 15 minutes, then hydrolysis of the substrate was stopped by the addition of 100µl of stop solution (0.4M sodium carbonate, 0.1M 3-(cyclohexylamino)-1-propanesulphonic acid, 0.1M ethylene diaminetetraacetic acid tetrasodium salt, 0.4M sodium hydroxide). Absorbance at 550nm was measured after the addition of the stop solution.

The results are given in Table 6 below.

TABLE 6

Absorbance at 550nm of βXIIa Standards and of Human Plasma Samples

Absorbance at 550nm	βXIIA Concentration in ng/ml	
0.0 0.0	0.0	Standard
0.047, 0.050	1.0	Standard
0.120, 0.118	2.5	Standard
0.264, 0.247	5.0	Standard
0.583, 0.556	10.0	Standard
1.549, 1.478	25.0	Standard
0.347, 0.314	6.4, 6.0	Sample
0.245, 0.244	4.8, 4.8	Sample
0.387, 0.376	7.0, 6.9	Sample
0.364, 0.300	6.6, 5.8	Sample
0.330, 0.310	6.2, 5.8	Sample
0.437, 0.457	7.8, 8.2	Sample
0.140, 0.143	2.8, 2.8	Sample
0.179, 0.173	3.6, 3.5	Sample
0.454, 0.436	8.2, 7.9	Sample
0.316, 0.302	6.0, 5.8	Sample

EXAMPLE 8

Assay using antibodies purified from polyclonal antiserum

The assay was carried out as described in Example 7 except that antibodies obtained from a polyclonal antiserum raised against Factor XII as described in Example 4 and subsequently conjugated to alkaline phosphatase as described in Example 6 were used instead of antibodies 201/9 and 202/16.1.9

The results are given in Table 7

TABLE 7

Absorbance at 550nm

βXIIa Concentration in ng/ml	Absorbance at 550nm
0	0.048, 0.054
10	0.209, 0.205
25	0.400, 0.396
50	0.564, 0.547
75	0.721, 0.689
100	0.774, 0.777
125	0.823, 0.816
150	0.823, 0.845
200	0.891, 0.911

EXAMPLE 9

Assay using a chromogenic substrate

100μl aliquots of βXIIa standards were added to the wells of microtitre plates that had been coated with monoclonal antibody 2/215 as described in Example 8. The plates were incubated at 20-25°C for 1 hour. Each well was then washed with the wash buffer (see Example 7) prior to the addition of 200μl of chromogenic substrate solution (2mM S2302 (Kabi Diagnostica, Uxbridge), 65mM Tris, 135mM sodium chloride). After incubation for 1 hour at 37°C, 50μl of 1% acetic acid was added to each well to stop the reaction. The absorbance at 405nm was measured using a microtitre plate reader.

The results are presented in Table 8.

TABLE 8

Absorbance at 405nm

βXIIa Concentration in ng/ml	Absorbance 405nm
0	0.058, 0.057
10	0.077, 0.072
50	0.148, 0.155
100	0.241, 0.226
150	0.275, 0.293
200	0.308, 0.300
250	0.310, 0.306

Note: This assay method is not accurate for βXIIa concentrations below about 10ng/ml, so is not sufficiently sensitive to determine βXIIa in plasma samples. Nevertheless, it is useful for assessing βXIIa at higher concentrations, for example, during isolation and purification and in resulting production batches.

CLAIMS:

1. A monoclonal antibody which binds to Factor βXIIa and which shows substantially no binding to Factor XII.

2. A monoclonal antibody as claimed in claim 1, which also binds to Factor αXIIa.

3. A monoclonal antibody as claimed in claim 1 or claim 2, having a corrected cross-reactivity with Factor XII of 0.1% or less.

4. A monoclonal antibody which is produced by hybridoma cell line 2/215 (ECACC 90011606) or by a reclone thereof, or by hybridoma cell line 201/9 (ECACC 90011893) or by a reclone thereof.

5. A monoclonal antibody as claimed in any one of claims 1 to 4, which has been provided with a detectable label.

6. A monoclonal antibody as claimed in any one of claims 1 to 4, immobilised on a solid support.

7. A hybridoma cell line which produces a monoclonal antibody as claimed in any one of claims 1 to 4, or a reclone thereof.

8. Hybridoma cell line 2/215 (ECACC 90011606) or a reclone thereof, or hybridoma cell line 201/9 (ECACC 90011893) or a reclone thereof.

9. A method of producing a monoclonal antibody as claimed in any one of claims 1 to 4, which comprises cultivating a hybridoma cell line capable of producing the antibody in a growth medium and obtaining the antibody from the

growth medium.

10. A method of producing a hybridoma cell line which produces a monoclonal antibody which binds to Factor βXIIa and which shows no substantial binding to Factor XII, which comprises administering an antigen to an animal to obtain antibody-producing cells, fusing the resulting antibody-producing cells with myeloma cells, and screening the resulting hybridomas for the production of the monoclonal antibody, wherein the antigen is Factor βXIIa or an antigenic fragment thereof.

11. A method of carrying out an immunoassay for an antigen in a sample of fluid, which assay comprises an interaction between an antigen and an antibody that binds thereto and determining the amount of antigen present in the sample with reference to results obtained using pre-determined amounts of a known antigen characterised in that the antibody is a monoclonal antibody as claimed in any one of claims 1 to 4, and the known antigen is Factor βXIIa.

12. A method of detecting and/or determining Factor XIIa or βXIIa in a sample, which comprises subjecting the sample to a qualitative or quantitative immunoassay which comprises the interaction between an antigen and an antibody and the detection and/or determination of any resulting antibody-antigen complex, characterised in that the antibody is a monoclonal antibody as claimed in any one of claims 1 to 4.

13. A method as claimed in claim 11 or claim 12, wherein the sample of fluid is a sample of plasma obtained from a human subject and wherein, to determine the susceptibility of the subject to heart disease, the result obtained for the level of the antigen in the sample assayed is compared with the results obtained in a large-scale investigation correlating the level of the antigen assayed with susceptibility to heart disease.

14. A kit for carrying out an immunoassay as claimed in any one of claims 11 to 13, which kit comprises, each in a separate container or otherwise compartmentalised, a monoclonal antibody as claimed in any one of claims 1 to 6, and Factor βXIIa or an antigenic fragment thereof.

15. A kit for carrying out an immunoassay as claimed in any one of claims 11 to 13, which kit comprises, each in a separate container or otherwise compartmentalised,

a)(i) a monoclonal antibody as claimed in any one of claims 1 to 4, optionally immobilised on a solid support, or

(ii) Factor βXIIa or an antigenic fragment thereof, or

(iii) an antibody directed against an antibody as claimed in any one of claims 1 to 4, optionally immobilised on a solid support;

b)(i) labelled antibody capable of reacting directly or indirectly with Factor βXIIa, or

(ii) labelled Factor βXIIa, or

(iii) a chromogenic substrate for Factor βXIIa; and

c) a control reagent, which is purified Factor βXIIa or an antigenic fragment thereof.

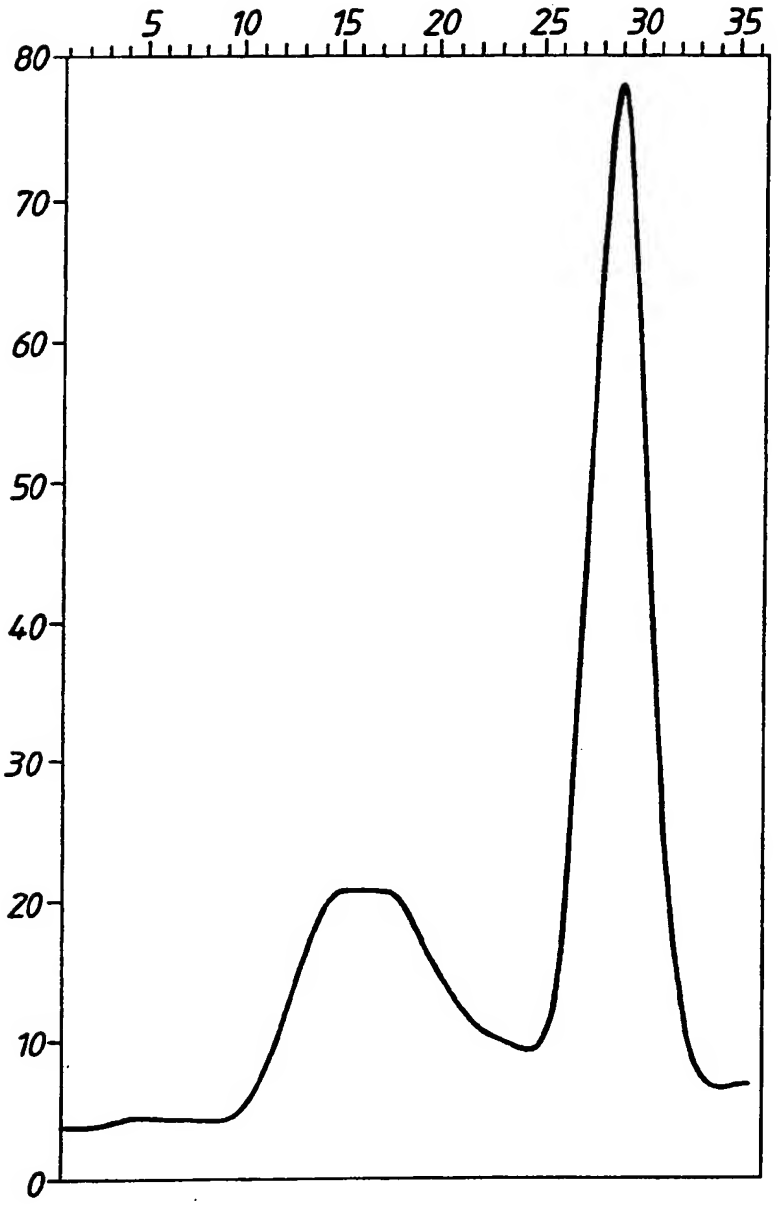

Fig.1.

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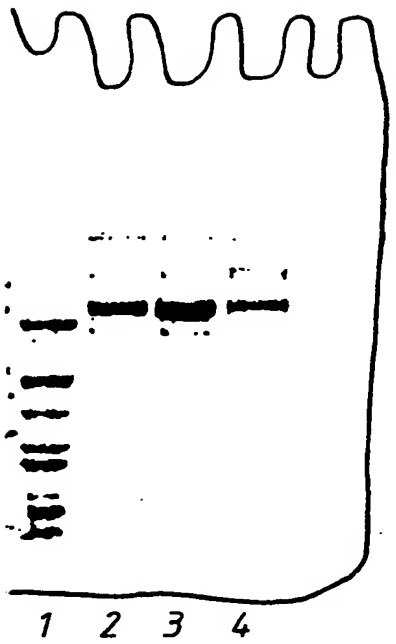

Fig. 2a.

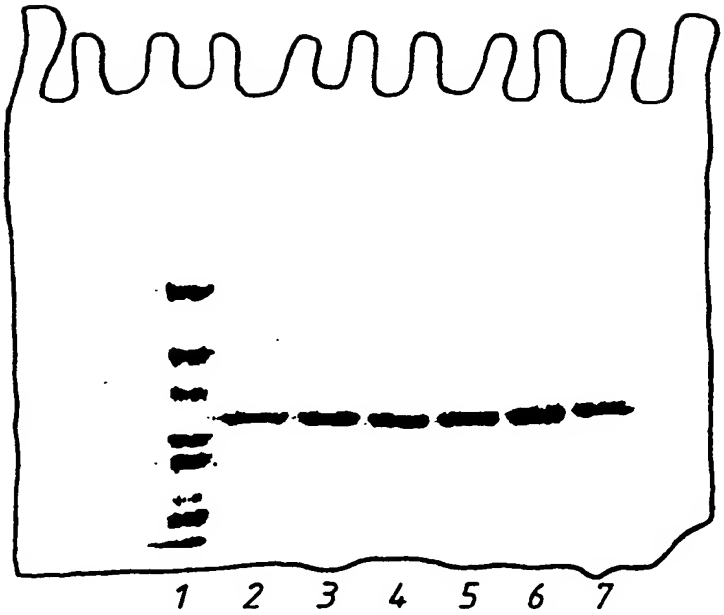

Fig. 2b.

SUBSTITUTE SHEET

3/3

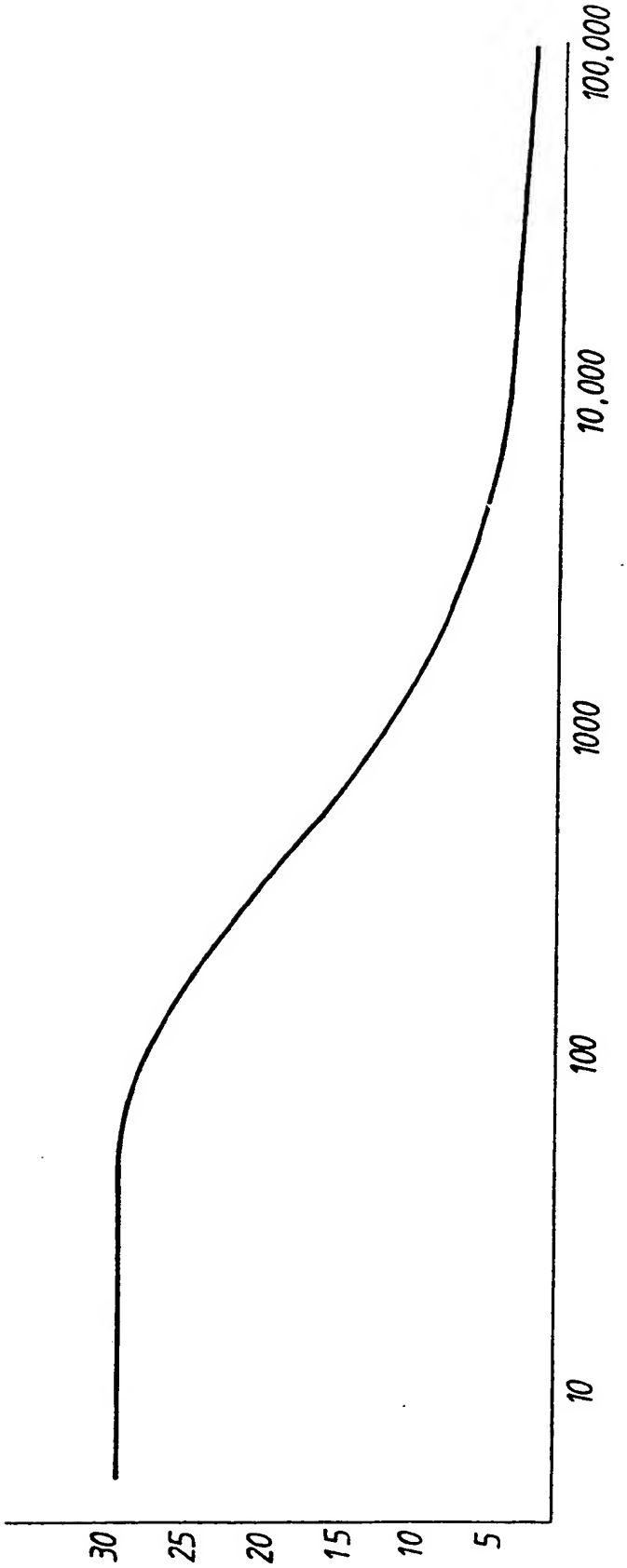

Fig.3.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 50, lines 9 & 10 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3] TWO FURTHER SHEETS ATTACHED

Name of depositary institution [4]

European Collection of Animal Cell Cultures

Address of depositary institution (including postal code and country) [4]

PHLS Centre for Applied Microbiology & Research,
Division of Biologics, Porton Down,
Salisbury, Wiltshire, SP4 0JG, United Kingdom.

Date of deposit [5]	Accession Number [6]
	90011606

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Date of deposit: 16 January 1990 (16.01.90)

E. [X] This sheet was received with the international application when filed (to be checked by the receiving Office)

25 JUN 1990

(Authorized Officer)

[X] The date of receipt (from the applicant) by the International Bureau [10]

[was 14/3/90]

[C. J. Noyce]

(Authorized Officer)

Form PCT/RO/134 (January 1981)

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 50, lines 11 & 12 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3] TWO FURTHER SHEETS ATTACHED

Name of depositary institution [4]

European Collection of Animal Cell Cultures

Address of depositary institution (including postal code and country) [4]

PHLS Centre for Applied Microbiology & Research,
Division of Biologics, Porton Down,
Salisbury, Wiltshire, SP4 0JG, United Kingdom.

Date of deposit [5]	Accession Number [6]
	90011893

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Date of deposit: 18 January 1990 (18.01.90)

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

25 JUN 1990

[X] The date of receipt (from the applicant) by the International Bureau [10]

[was 14/3/90]

[ ] (Authorized Officer)

[G. J. Noyce] (Authorized Officer)

Form PCT/RO/134 (January 1981)

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 50, line S 13 & 14 of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet [X] [3] TWO FURTHER SHEETS ATTACHED

Name of depositary institution [4]

European Collection of Animal Cell Cultures

Address of depositary institution (including postal code and country) [4]

PHLS Centre for Applied Microbiology & Research,
Division of Biologics, Porton Down,
Salisbury, Wiltshire, SP4 0JG, United Kingdom.

Date of deposit [5]	Accession Number [6]
	90012512

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (If the indications are not for all designated States)

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Date of deposit: 25 January 1990 (25.01.90)

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

25 JUN 1990 (Authorized Officer)

[X] The date of receipt (from the applicant) by the International Bureau [10]

[was 14/3/90] [G. J. Noyce] (Authorized Officer)

Form PCT/RO/134 (January 1981)

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00129

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, G 01 N 33/573, 33/68, 33/86

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 P; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 89/11865 (TEMPLE UNIVERSITY OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 14 December 1989, see the whole document --	1-10
X	Chemical Abstracts, volume 108, no. 5, 1 February 1988, (Columbus, Ohio, US), J.H. Nuijens et al. : "Detection of activation of the contrat system of coagulation in vitro and in vivo: quantitation of activated Hageman factor-C1-inhibitor and kallikrein-C1-inhibitor complexes by specific radioimmunoassays", see page 273, abstract 33870k, & Thromb. --	1,6,7,9-12,15

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th April 1990	10. 05. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	Chemical Abstracts, volume 111, no. 13, 25 September 1989, (Columbus, Ohio, US), J.H. Nuijens et al. : "Activation of the contact system of coagulation by a monoclonal antibody directed against a neodeterminant in the heavy chain region of human coagulation factor XII (Hageman factor) ", see page 438, abstract 112739q, & J. Biol. Chem. 1989, 264(22), 12941-9 --	1
A	Chemical Abstracts, volume 107, no. 15, 12 October 1987, (Columbus, Ohio, US), Robin A. Pixley et al. : "A monoclonal antibody recognizing an icosapeptide sequence in the heavy chain of human factor XII inhibits surface-catalyzed activation ", see page 340, abstract 129916k, & J. Biol. Chem. 1987, 262(21), 1014- 5 --	1,9, 11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/00129

SA 34241

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/03/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 89/11865	14/12/89	NONE	

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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